Original Article

HLA Class I Expressions on Peripheral Blood Mononuclear Cells in Colorectal Cancer Patients

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ABSTRACT

Objective: To investigate the expression change of human leukocyte antigen (HLA) class I on human peripheral blood mononuclear cells (PBMCs) at both mRNA and protein levels, and to evaluate its roles in the development of colorectal cancer (CRC).

Methods: In the present study, 50 patients with CRC, 35 patients with benign colorectal lesion and 42 healthy volunteers were enrolled. Expression levels of HLA class I mRNA and protein were determined using real-time quantitative reverse transcription PCR (RT-PCR) and flow cytometry analysis, respectively.

Results: The expression levels of HLA class I mRNA and proteins were not influenced by age and gender. The relative ratios of HLA class I mRNA were 0.99±0.27 in healthy controls, 0.76±0.19 in benign patients, and 0.48±0.21 in CRC patients. Mean fluorescence intensities of HLA class I were 145.58±38.14 in healthy controls, 102.05±35.98 in benign patients and 87.44±34.01 in CRC patients. HLA class I on PBMCs was significantly down-regulated at both mRNA and protein levels in patients with stage III and IV CRC. CRC patients with lymph node metastasis also showed a decreased HLA class I expression at protein level.

Conclusion: HLA class I expressions on PBMCs are associated with staging of CRC and lymph node metastasis. Monitoring the expression of HLA class I on PBMCs may provide useful information for diagnosis and metastasis judgement of CRC.

Key words: HLA class I; Peripheral blood mononuclear cells; RT-PCR; Flow cytometry; Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors. In the USA, CRC is the third most frequently diagnosed cancer in men and the second in women. Deaths from CRC rank third after lung and prostate cancers for men and third after lung and breast cancers for women. The incidence of CRC in China is lower than that in west countries, but has increased in recent years and become a substantial cancer burden. Therefore, it is important to prevent, detect, and treat CRC early enough.

There is a significant difference in survival rates between patients with early-stage CRC and those with advanced CRC[1, 2]. Thus, early diagnosis of CRC is imperative for obtaining a better therapeutic outcome, but still remains a challenge though promising advances in imaging technology and other diagnostic methods have been achieved in recent years.

Human leukocyte antigens (HLA) are cell surface glycoproteins that play critical roles in the regulation of immune responses. These molecules are expressed on the surface of all nucleated cells, necessary for the presentation of peptide antigens to cytotoxic T lymphocytes (CTLs)[3] and for the immune regulatory activity exerted by NK cells[4]. It is widely accepted that total or partial loss of HLA class I molecules on tumor cells was one of the main mechanisms of tumor escape. Studies have demonstrated that HLA class I molecules had the ability to control the metastatic activities of tumor cells[5-7], and had a close

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relationship with patients' prognosis[8, 9], and their polymorphisms influenced tumor susceptibility[10, 11].

Past researches paid more attention to HLA class I on the surface of tumor cells. There are plentiful HLA class I molecules on the surface of peripheral blood mononuclear cells (PBMCs), however, what happens to these molecules during the development of CRC remains unclear. In the present study, we enrolled CRC patients, benign colorectal diseases patients and healthy individuals from Qilu Hospital, collected their peripheral blood and measured HLA class I levels at both mRNA and protein levels, in an attempt to investigate the expression changes of HLA class I during the development of CRC.

MATERIALS AND METHODS

Patients

Fifty CRC patients (21 female and 29 male, age range 56–77 years, median 62 years) were enrolled in this study (Table 1). Enrollment took place between Feb 2008 and Oct 2010 at the Department of General Surgery, Qilu Hospital, Jinan, China. Clinical stages and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer (AJCC). None of these patients had been treated with radiotherapy or chemotherapy prior to enrollment. Benign group of colorectal diseases consisted of 35 patients (12 female and 23 male, age range 52–78 years, median 60 years), including 12 proctitis, 7 ulcerative colitis and 16 colonic polyps. Control volunteers were from the Department of Health Examination Center, and consisted of 42 healthy adults (20 female and 22 male, age range 56–79 years, median 63 years). All subjects complicated with hepatitis B virus (HBV) infection, hepatic cirrhosis, hepatic cancer[12], common cold or influenza were excluded for their potential interference to the expression of HLA class I. Informed consent was obtained from each participating patient. Ethical approval for this study was obtained from the Medical Ethical committee of Qilu Hospital, Shandong University.

RNA Extraction and Reverse Transcription (RT)

Blood (2 ml) was drawn into sterile heparinized tubes from each patient and control. The blood was centrifuged and heparinized plasma was stored at –80°C until determination of CEA and CA 19-9. Mononuclear cells were isolated from heparinized blood by gradient centrifugation on Ficoll-Hipaque (Haoyang, Tianjin, China). Total RNA was extracted from PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was obtained using a PrimeScript™ reverse transcriptase reagent kit (Takara, Dalian, China) according to the manufacturer's instructions.

Table 1. Demographic and clinicopathological characteristics of CRC patients

Indices	*n*
Age (year)	
<60	15
≥60	35
Gender	
Male	29
Female	21
Tumor size*	
<2 cm	22
≥2 cm	28
Lymph node metastasis	
Absent	17
Present	33
TNM stage	
I	6
II	13
III	24
IV	7

*Tumor size was measured for invasive area by histological examination.

HLA Class I mRNA Expression Analysis

The expression of HLA class I mRNA was measured by relative quantitative real-time polymerase chain reaction (PCR) using a SYBR Premix Ex Taq™ II kit (Takara) and the ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Fold expression changes were determined by the $2^{-\Delta\Delta CT}$ method[13]. The primer sequences for HLA class I mRNA were: forward primer 5′-CCTACG-ACGGCAAGGATTAC-3′, reverse primer 5′-TGCC-AGGTCAGTGTGATCTC-3′. The primer sequences for endogenous control (beta-actin) were: forward primer 5′-TTGCCGACAGGATGCAGAA-3′, reverse primer 5′-GCCGATCCACACGGAGTACT-3′. The PCR cycling conditions were: initial reaction at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and finally 60°C for 34 s. All reactions were performed in triplicate.

Flow Cytometry

Heparinized peripheral blood samples (2 ml of each) were taken from all subjects. An amount of 100 μl blood samples was added to polystyrene test tubes (Becton Dickinson, NJ, USA), and then monoclonal antibodies (mAbs) against HLA class I (HLA-I-PE-Cy5, Becton Dickinson) were added. All stainings were conducted under saturating concentrations of mAbs. After an incubation time of 30 min in darkness, red

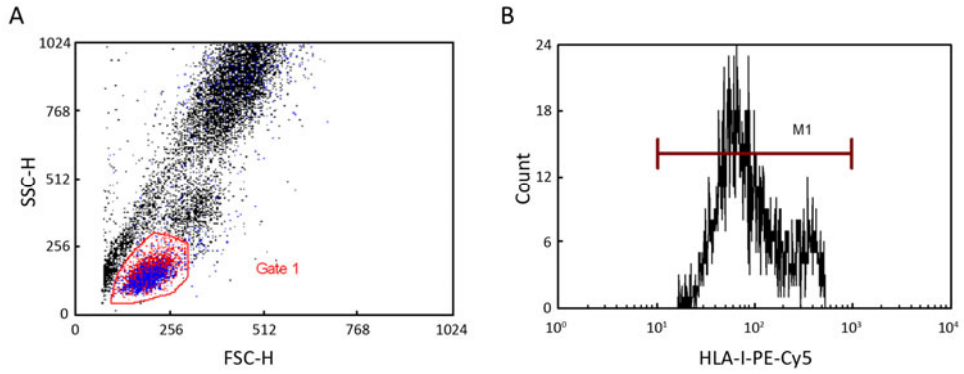

Figure 1. The gates of PBMCs and HLA class I expression on PBMCs by flow cytometry. **A**: PBMCs were separated from peripheral blood (Gate 1); **B:** Mean fluorescence intensity of HLA class I on PBMCs was measured, and the area under the curve represented the expression levels of HLA class I.

blood cells were lysed by FACS lysing solution (Becton Dickinson) for 10 min at room temperature in the dark. And cells were washed twice and resuspended in 400 μl phosphate-buffered saline (PBS) and then analyzed with an FACS flow cytometer (Becton Dickinson).

The cell samples were run through the flow cytometer, and 10,000 events were analyzed for each sample. Using the forward- and side-scatter (FSC and SSC) properties of cells in laser light, a gate was drawn around the cells to exclude other nucleated cells for further analysis (Figure 1). The mean fluorescence intensity of HLA class I on PBMCs was calculated using FCS Express™ V3 for Windows XP. Negative and isotypic controls were performed routinely.

Statistical Analysis

Data were expressed as $\bar{x}\pm s$ and analyzed using Statistical Package for the Social Sciences version 11.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for comparison of differences among groups, and least significant difference (LSD) for comparison between groups. A probability value, $P<0.05$, was considered statistically significant.

RESULTS

Peripheral blood was collected from 50 CRC patients, 35 patients with benign colorectal diseases and 42 healthy volunteers following a standardized procedure. The demographic data and clinico-pathological characteristics of all CRC patients are summarized in Table 1.

For the determination of HLA class I mRNA, we designed the primers for the conservative region. The length of amplification product is 305 bp (Figure 2). The amplification ratios of HLA class I and beta-actin were 0.96 and 0.95, respectively. Thus, it is suitable to use $2^{-\Delta\Delta CT}$ method to analyze mRNA levels of HLA class I. As shown in Table 2, HLA class I in CRC patients was lower than that in healthy volunteers at both mRNA and protein levels. And HLA class I molecule on PBMCs in patients with benign colorectal diseases was lower than that in healthy controls.

As shown in Table 3, age, gender and tumor size did not affect HLA class I expressions at both mRNA and protein levels. However, CRC patients with lymph node metastasis had a lowered expression of HLA class I molecules. Expression of HLA class I in patients with stage II to stage IV CRC was lower than that of stage I patients at both mRNA and protein levels.

Table 2. Expressions of HLA class I in CRC patients, benign colorectal diseases patients and healthy controls

Groups	HLA class I mRNA	HLA class I protein
Healthy controls	0.99±0.27	145.58±38.14
Benign diseases	0.76±0.19	102.05±35.98*
CRC	0.48±0.21$^{*\#}$	87.44±34.01$^{**\#}$

$^{*}P<0.05$, $^{**}P<0.01$, compared with health controls; $^{\#}P<0.05$, compared with benign diseases patients.

DISCUSSION

HLA class I molecules are highly polymorphic molecules, essential for the presentation of endogenous peptides to T cells. It is hard to detect the expression of every HLA class I allele due to the high polymorphism. In the present study, we designed specific PCR primers for HLA class I mRNA. The sense primer used, corresponding to the α3 extracellular domain of HLA class I molecules, was encoded by the fourth exon. The antisense primer, corresponding to the transmembrane part of HLA-I molecules, was encoded by the fifth exon. This RT-PCR method for HLA class I mRNA detection was validated by electrophoresis of PCR products and

melting curve of real-time quantitative RT-PCR, suitable for the measurement of HLA class I mRNA. The mAb used here reacts with a monomorphic epitope of HLA class ABC molecules and is routinely tested by flow cytometric analysis by BD Pharmingen™.

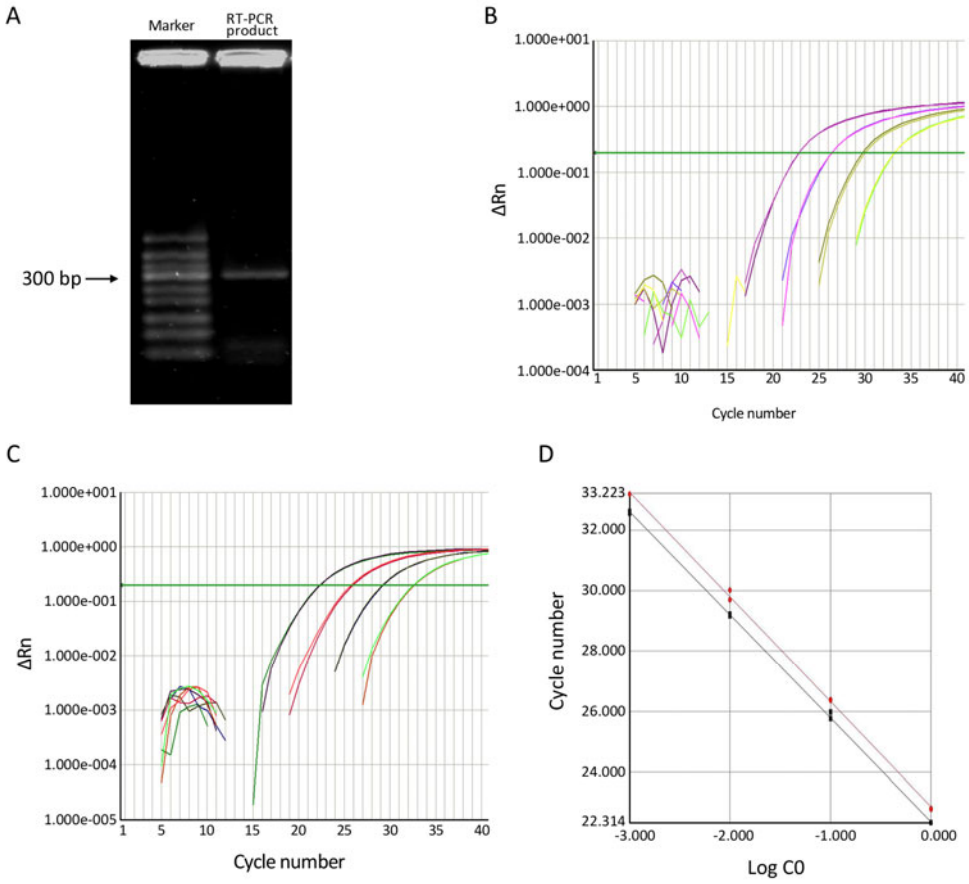

Figure 2. RT-PCR analysis of HLA class I mRNA. Beta-actin was used as internal control. **A:** Electrophoresis of RT-PCR product of HLA class I mRNA; **B:** Identify-cation of linear range of RT-PCR of HLA class I mRNA; **C:** Identification of linear range of RT-PCR of beta-actin mRNA; **D:** Standard curve of HLA class I and beta-actin.

Table 3. Correlation between the expression of HLA class I on PBMCs and clinicopathological characteristics

Indices	HLA class I mRNA	HLA class I protein
Age (year)		
<60	0.98±0.35	119.76±28.35
≥60	0.90±0.28	103.71±31.26
Gender		
Male	0.99±0.29	121.52±31.49
Female	1.05±0.32	104.16±37.46
Tumor size		
<2 cm	0.98±0.41	87.10±21.65
≥2 cm	0.89±0.39	83.76±19.29
Lymph node metastasis		
Absent	1.01±0.26	101.30±32.69
Present	0.78±0.35	72.91±29.18*
TNM stage		
I	1.10±0.29	98.08±21.62
II	0.87±0.23	89.29±27.26
III	0.46±0.31#	61.49±18.52†
IV	0.39±0.35#	54.61±29.81†

*P<0.05, compared with absent lymph node metastasis patients; #P<0.05, compared with stage I CRC patients; †P<0.05, compared with stage I CRC patients.

Our previous studies demonstrated that in gastric cancer[14], hepatocellular cancer[12], esophageal cancer[15], and breast cancer[16], HLA class I on PBMCs was down-regulated and associated with stages of tumors. In the present study, CRC patients had down-regulated expression of HLA class I at both mRNA and protein levels, and in patients with stage II to IV CRC, the down-regulation tendency was particularly significant. These findings indicated that the down-regulation of HLA class I on PBMCs in tumors above is common, and that expression changes of HLA class I on PBMCs may reflect the existence of tumors. In the present study, CRC patients with lymph node metastasis had a decreased expression of HLA class I protein compared with those without lymph node metastasis, which might be associated with the fact that tumor cells break out through the basement membrane, release into blood massively. Patients with benign colorectal diseases also had a lowered HLA class I protein. The reason might be that many benign colorectal diseases, such as polyp, are considered as precancerous lesions[17, 18]. Of course, further follow-up is imperative to observe the morbidity of these patients with benign colorectal diseases whose HLA class I on PBMCs are down-regulated.

Several HLA class molecules are expressed on the surface of peripheral T lymphocytes. These molecules had the ability to protect T cells from deletion mediated by antibody and macrophage[19]. When masking these HLA class I molecules with specific mAb or antibody Fab fragments, this resistance collapses, suggesting that high-expressed HLA class I molecules contributed to prolong survival time of T cells. *In vitro* RNA interference studies showed that inhibition of H-2K^d expression in mouse reduced the cytotoxicity of LAK cells to H22 and K562 cells, indicating that HLA class I expression levels on LAK cells affected their cytotoxicity[20]. In patients infected with HBV, the killing activity of peripheral blood lymphocytes decreased with the expression of HLA class I on their cell surface. This decrease was most significant in patients with hepatocellular carcinoma[12]. Down-regulation of HLA class I molecules was common in tumors mentioned above, and may shorten survival time of peripheral T lymphocytes and lower their killing activity, facilitating development of tumors. CRC patients with lymph node metastasis also showed a decreased HLA class I expression at protein level, suggesting that regulation at post-transcriptional levels may be involved in modulating the expression of HLA class I molecules on PBMCs.

Nowadays, HLA class I molecules on peripheral blood T cells were considered as an accurate and reliable predictor of acute rejection[21, 22] after organ transplantation. Our finding demonstrated that HLA class I molecules on PBMCs were down-regulated in many tumors, suggesting this parameter represents host immune status to some extent. Detection of HLA class I on PBMCs is cheap, simple, convenient and non-invasive, and this parameter may provide valuable information for host immune status and lead to new insight into tumor immunity.

REFERENCES

1. Wang HZ, Huang XF, Wang Y, et al. Clinical features, diagnosis, treatment and prognosis of multiple primary colorectal carcinoma. World J Gastroenterol 2004; 101:2136–9.
2. Zhang YL, Zhang ZS, Wu BP, et al. Early diagnosis for colorectal cancer in China. World J Gastroenterol 2002; 8:21–5.
3. Townsend AR, Rothbard J, Gotch FM, et al. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 1986; 44:959–68.
4. Ljunggren HG, Kärre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today 1990; 11:237–44.
5. Plaksin D, Gelber C, Feldman M, et al. Reversal of the metastatic phenotype in Lewis lung carcinoma cells after transfection with syngeneic H-2Kb gene. Proc Natl Acad Sci USA 1988; 85:4463–7.
6. Eisenbach L, Hollander N, Greenfeld L, et al. The differential expression of H-2K versus H-2D antigens, distinguishing high-metastatic from low-metastatic clones, is correlated with the immunogenic properties of the tumor cells. Int J Cancer 1984; 34: 567–73.
7. Porgador A, Feldman M, Eisenbach L. H-2Kb transfection of B16 melanoma cells results in reduced tumourigenicity and metastatic competence. J Immunogenet 1989; 16:291–303.
8. Simpson JA, Al-Attar A, Watson NF, et al. Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. Gut 2010; 59:926–33.
9. Benevolo M, Mottolese M, Piperno G, et al. HLA-A, -B, -C expression in colon carcinoma mimics that of the normal colonic mucosa and is prognostically relevant. Am J Surg Pathol 2007; 31:76–84.
10. Kabbaj M, Oudghiri M, Naya A, et al. HLA-A, -B, -DRB1 alleles and haplotypes frequencies in Moroccan patients with leukemia. Ann Biol Clin 2010; 68:291–6.
11. Hu SP, Zhou GB, Luan JA, et al. Polymorphisms of HLA-A and HLA-B genes in genetic susceptibility to esophageal carcinoma in Chaoshan Han Chinese. Dis Esophagus 2010; 231:46–52.
12. Wang CX, Wang JF, Liu M, et al. Expression of HLA class I and II on peripheral blood lymphocytes in HBV infection. Chin Med J 2006; 119:753–6.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402–8.
14. Zhang Y, Liu Y, Lu N, et al. Expression of the genes encoding human leucocyte antigens-A, -B, -DP, -DQ and -G in gastric cancer patients. J Int Med Res 2010; 38:949–56.
15. Li H, Zou X, Zhao SM, et al. Expressions of HLA-A and B mRNA in peripheral blood mononuclear cells in patients with esophagus cancer of the neck. Shan Dong Da Xue Er Bi Hou Yan Xue Bao (in Chinese) 2009; 23:8–11.
16. Zhao S, Yang X, Lu N, et al. the amount of surface HLA-I on T lymphocytes decreases in breast infiltrating ductal carcinoma patients. J Int Med Res 2011; 39:508–13.

17. Jass JR, Baker K, Zlobec I, et al. Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. Histopathology 2006; 49:121–31.
18. Chan TL, Zhao W, Leung SY, et al. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res 2003; 63:4878–81.
19. Orlikowsky T, Wang Z, Dudhane A, et al. Elevated major histocompatibility complex class I expression protects T cells from antibody- and macrophage-mediated deletion. Immunology 1998; 95:437–42.
20. Shan NN, Zou X, Yang XJ, et al. Silence of H-2Kd gene by antisense oligonucleotides complementary has effect on the cytotoxicity of LAK cells in mouse. Zhong Hua Jian Yan Yi Xue Za Zhi (in Chinese) 2004; 27:327–9.
21. Blanco-Garcia RM, López-Alvarez MR, Pascual-Figal DA, et al. Expression of HLA molecules on peripheral blood lymphocytes: a useful monitoring parameter in cardiac transplantation. Transplant Proc 2007; 39:2362–4.
22. Tian J, Shi WF, Zhang LW, et al. HLA class I (ABC) upregulation on peripheral blood CD3+/CD8+ T lymphocyte surface is a potential predictor of acute rejection in renal transplantation. Transplantation 2009; 88:1393–7.